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Synthesis of Surfactin by *Bacillus subtilis* MTCC 2423 from waste fried oils

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ABSTRACT

Biosurfactant production by *Bacillus subtilis* MTCC 2423 was studied in shake flasks, using waste fried sunflower oil and waste fried rice bran oil as substrates and compared with the biosurfactant produced by conventional substrate, 'glucose'. Reduction in surface tension and increase in cell bio mass were observed for a period of five days. *Bacillus* growing in waste fried sunflower oil decreased the surface tension by 55% whereas conventional substrate glucose produced a reduction of 68%. Cell biomass formation was more with waste fried rice bran oil, compared to waste fried sunflower oil. Attempts were made to extract biosurfactants by solvent extraction. Our studies have shown that waste fried oil, which is abundantly available at cheaper costs, can be effectively used as carbon source to produce biosurfactants, a high value bio product.

Key words: Biosurfactants, *Bacillus subtilis*, Surface Tension, Waste fried oils, Secondary metabolite

INTRODUCTION

Surfactants are substances that absorb to and alter the conditions prevailing at interfaces, as they are amphipathics containing both hydrophobic and polar groups [1]. These amphipathic molecules partition preferentially at the interface between two phases of different degrees of polarity and hydrogen bonding such as oil and water or air and water interface [2]. Surfactants reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface.

The effectiveness of a surfactant is determined by its ability to lower surface tension, which is a measure of the surface free energy per unit area required to bring a molecule from the bulk phase to the surface [3]. When surfactant is added to oil and water systems at increasing concentrations, a reduction of surface tension is observed to a critical value, which is known as critical micelle concentration. It is the solubility of a surfactant within an aqueous phase and is used to measure the efficiency of a surfactant. Current world wide production of surfactants exceeds four million tonnes per annum and is expected to rise to over ten million tonnes.

Unfortunately majority of these chemical surfactants are environmentally objectionable compounds. Increased environmental awareness and strict legislation has made environmental compatibility of surfactants an important factor in their application for various uses.

The unique properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in a great number of industrial applications [4].

Surfactants are used by many industries and one could easily say that there is almost no modern industrial operation where properties of surfactants are not exploited.

Biosurfactants have become recently an important product of biotechnology for industrial and medical applications. The reason for their popularity, as high value microbial products, is primarily in their specific action, low toxicity, relative ease of preparation and widespread applicability [5].

They can be used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors such as petroleum and petrochemicals, organic chemicals, foods and beverages, cosmetics and pharmaceuticals, mining and metallurgy, agrochemicals and fertilizers, environmental control and many others [6].

Unlike chemically synthesized surfactants, which are classified according to the nature of their polar grouping, biosurfactants are categorized mainly by their chemical composition and their microbial origin. Basically there are six major types of biosurfactants viz., hydroxylated and cross linked fatty acids,

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glycolipids, lipopolysaccharides, lipoproteins-lipopeptides, phospholipids and the complete cell surface itself [7].

A survey of literature shows that biosurfactants are produced by a wide variety of microorganisms, how ever the chemical nature of biosurfactant is dependent on the producing species [8]. Among the biosurfactant producing potential microbes, *Bacillus subtilis* are known to produce cyclic lipopeptides including surfactins, iturins, fengycins, and lichenysins as the major classes of biosurfactants.

The history of surfactin dates back to 1968, when first time the presence of a new biologically active compound in the culture broth of *Bacillus subtilis* strain was reported. It was named 'surfactin' because of its exceptional surfactant activity and its structure was elucidated as that of a macrolide lipopeptide. Surfactin's structure consists of a peptide loop of seven amino acids and a hydrophobic fatty acid chain with thirteen to sixteen carbons.

The seven amino acids are L-glutamine, L-leucine, D-leucine, L-valine, L-asparagine, D-leucine, and L-leucine. Hydrophobic amino acid residues leucine, valine are located at positions 2,3,4,6 and 7 while the hydrophilic glutamyl and aspartyl residues are at position 1 and 5 respectively. Because of this structure, surfactin is one of the most powerful biosurfactant, which is capable of lowering the surface tension of water from 72 $mN.m^{-1}$ to 22 $mN.m^{-1}$ at a concentration as low as 10 µM.

The ability of surfactin synthesis is widely distributed not only in *Bacillus subtilis* but also among *Bacillus pumilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* strains [2]. Surfactin is produced in a nucleic acid independent way though the use of large enzyme complexes called surfactin synthetase complex. This complex consists of four enzymatic subunits, SrfA, SrfB, SrfC and SrfD [9].

The non ribosomal machinery for peptide synthesis uses these complexes as an assembly line to catalyze stepwise peptide condensation. The substrates are not restricted to the 20 amino acids, since hundreds of building blocks are now known to be integrated and modified by post synthetic action.

Typical of this assembly line is the incorporation of nonproteinogenic amino acids, such as D-isomers, carboxy acids and N- methylated residues, as well as the incorporation of heterocyclic rings and fatty acids [2].

Economy is often the bottleneck of biotechnological processes, especially, in the case of biosurfactant production. There are four factors that should be focused on to reduce biosurfactant production cost. The microbes (selected, adapted or engineered for higher yields of the product), the process, the substrate and process byproducts.

The choice of inexpensive raw materials is important to the overall economics of the process because they account for nearly 50% of the final product cost [10]. Few attempts at using waste for biosurfactant production and only a few types of biosurfactants produced from waste have been reported.

Oils and fats are found in all living cells. World production of oils and fats is about 80 Million tonnes, 80% of which are derived from plants. Most of the oils and fats are used in the food industry which generates great quantities of wastes.

The disposal of these wastes is a growing problem which explains the increasing interest in the use of wastes for microbial transformation. In this paper, a study has been made on the reuse of waste fried sun flower oil and waste fried rice bran oil as substrates for surfactin production using *Bacillus subtilis* bacteria.

MATERIALS AND METHODS

Bacillus subtilis MTCC 2423 was employed for biosurfactant production. It was procured from Microbial Type Culture Collection (MTCC), Institute of microbial Technology, Chandigarh, India.

The culture was maintained on nutrient agar plates, as per the recommendations of the supplier and stored in agar slants at 4° C for further use. Waste fried sunflower oil and waste fried rice bran oil were chosen as substrates for the study and were procured from a local restaurant. The choice of substrates was driven by the fact that both are non conventional sources which are easily available, inexpensive, easy to handle.

The culture was grown in a 100 ml Erlenmeyer flask on nutrient broth obtained from HiMedia, Mumbai, India for 24 hours at 30˚C. This was used as inoculum at 5% level (O.D. at 600 nm was 8.2 to 8.9). Experiments were conducted in 250 ml Erlenmeyer flasks containing 50 ml medium.

The concentration of the medium was (g/l): glucose/waste sunflower oil/waste rice bran oil 50, yeast extract 5, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5, $CaCl_2$ 0.1, NaCl 0.1, and Peptone 0.7. Three separate flasks one with glucose as control and the other two with waste sunflower oil and waste rice bran oil were prepared and sterilized at 121˚C. The final pH was adjusted to 7.2.

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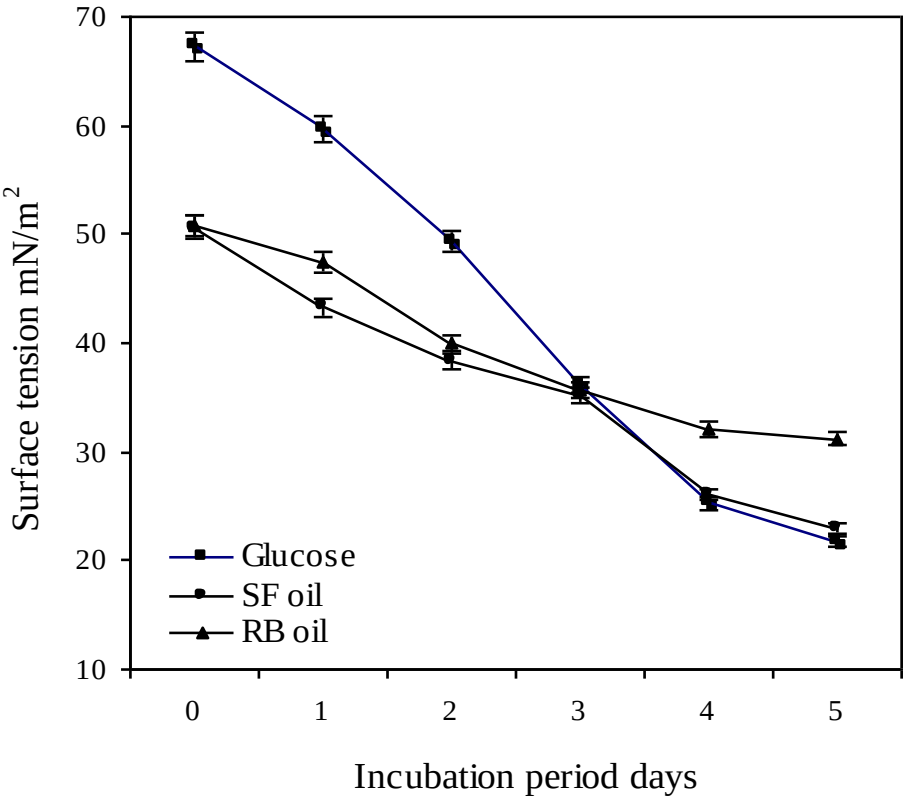

Fig. 1 Reduction in surface tension of cell free culture broth

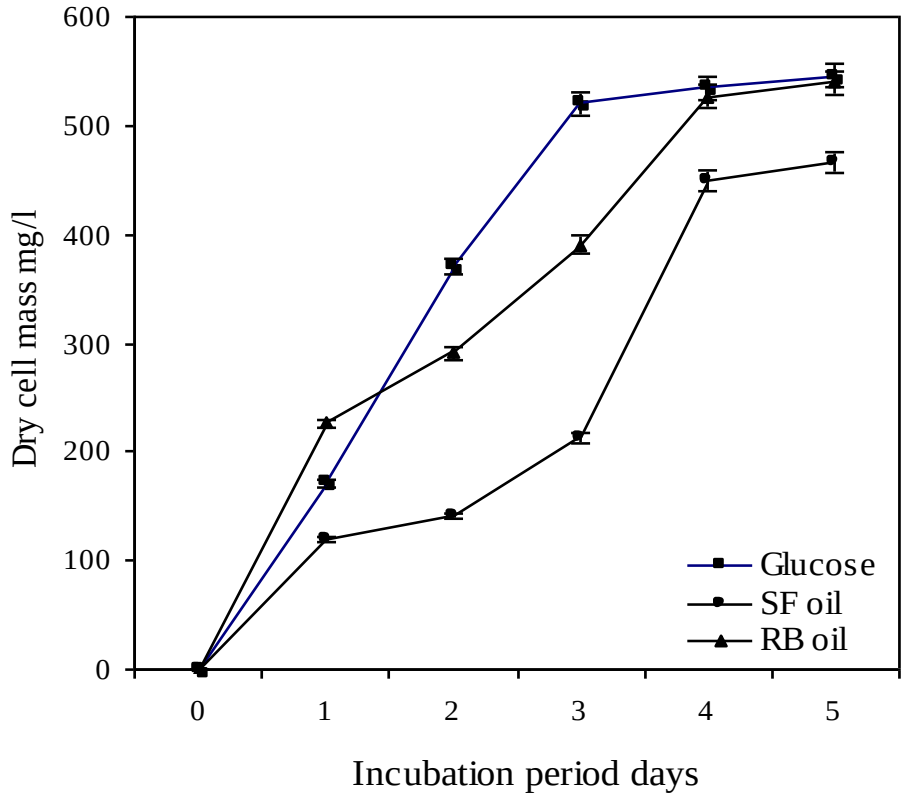

Fig. 2 Increase in Dry cell Mass

After adding the inoculum, the flasks were incubated on a rotary shaker at a speed of 160 rpm. Reduction in surface tension and increase in cell bio mass were observed everyday for a period of five days. Surface tension measurements were made by the du Nouy-ring method using Surface tension Balance – Interfacial tensiometer with 4 cm platinum ring as per Indian standards methods ((IS) 6104 and American Society for Testing and Materials (ASTM) D 971 specifications) at room temperature.

A platinum wire ring was placed into the solution and then slowly pulled through the liquid-air interface. Stabilization was allowed to occur until a standard deviation of 10 successive measurements was less than 0.4 mN/m. Between each measurement, the platinum ring was rinsed with water and acetone and allowed to dry.

The liquid from the culture was taken once in a day and centrifuged at 6000 rpm and the cell pellet obtained is oven dried at 105˚C over night and then weighed, to quantify the crude dry cell bio mass. This was done in duplicate and the average value was recorded. At the end crude surfactin was recovered by acid precipitation. 0.1 M hydrochloric acid was used to reduce the pH of the cell free fermentation broth to 2 and kept overnight at 4˚C. The precipitated biosurfactant was separated, dried and weighed [11].

RESULTS AND DISCUSSION

It has been shown previously that surfactin is synthesized by *Bacillus* during the stationary phase when nutrients in the culture media are limited [2]. From the biomass formation data, it was clear that by fourth day, cells have entered stationary phase. Hence the influence of incubation time on biomass formation and reduction in the surface tension were observed for over five days, and are represented in the figures 1 and 2.

The reduction in the surface tension was quite rapid after two days. The surface tension of cell free culture broth with glucose substrate after five days was 22.7 mN/m^2. For cell free culture broths with sunflower oil and rice bran oil the reduction in surface tension was 22.9 and 31.2 mN/m^2, respectively. Cell growth attained stationery phase slowly in the case of waste sun flower oil and waste rice bran oil due to the fact that *Bacillus* took more time for acclimatization.

At the end of incubation period, glucose substrate produced 546 mg/l of dry cell mass, where as sunflower oil and rice bran oil produced 466 mg/l and 540 mg/l dry cell mass respectively. 1.8 g/l of crude surfactin was recovered by acid precipitation, with glucose substrate. 1.52 g/l of crude surfactin was recovered with waste sunflower oil as substrate. Surfactin obtained was only 1.2 g/l, with waste rice bran oil substrate.

CONCLUSIONS

A major obstacle on the way of wide scale industrial application of biosurfactant is the high production cost coupled with less production rate. To reduce production cost, it is necessary to use cost-free or low cost feed stocks.

Thousands of liters of cooking oil is used around the world which ultimately produces huge amounts of waste fried oils whose disposal is a great problem for the restaurants and catering units.

Our investigations have clearly shown that the waste sunflower oil and waste rice bran oil can be effectively used as substrates for the production of the biosurfactants. *Bacillus* growing in waste fried sunflower oil decreased the surface tension by 55% whereas conventional substrate glucose produced a reduction of 68%.

The surface tension reduction by the surfactin produced by *Bacillus* in rice bran medium was about 39%. Cell bio mass growth was more with waste fried rice bran oil. Even though purity of the product obtained was inferior, compared to conventional substrates, crude biosurfactant itself can be directly utilized in most applications related to both the oil industries and bioremediation.

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